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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02018495.8

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Non-glycosylated polyacrylamide conjugates and their use for cytoprotection

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NON-GLYCOSYLATED POLYACRYLAMIDE CONJUGATES AND THEIR USE FOR CYTOPROTECTION

Field of the invention

The present invention relates to new non-glycosylated polyacrylamide conjugates, a method for protecting endothelial cells from complement-mediated cytotoxicity and the use of said non-glycosylated polyacrylamide conjugates as a medicament.

Background of the invention

Complement mediated damage of - mainly endothelial - cells plays an important role in a number of different pathophysiological processes, among which are acute vascular rejections of allo- and xenografts, ischemia/reperfusion injury (I/R injury), severe sepsis or septic shock, and arteriosclerosis. One example from the clinical practice is organ transplantation, in which no satisfactory therapeutic options are available to prevent and/or treat the I/R injury of the graft, nor to treat acute vascular rejection reactions that may occur due to preformed antibodies. In both cases complement-mediated damage to mainly endothelial cells plays a major role. In the case of HLA-immunized recipients or in ABO-incompatible- and xenotransplantation, specific antibodies bind to the graft endothelium and are responsible for activation of the complement system and subsequently the endothelial cells themselves. In I/R injury the ischemia during storage of the graft leads to changes on the endothelium that render it susceptible to complement– and coagulation–mediated damage upon reperfusion. The use of soluble complement inhibitors / endothelial cell protectants might therefore be a valid therapeutical approach for both clinical problems.
To date, depletion of the central complement component C3 by cobra venom factor is the most efficient experimental treatment, but results in the loss of major defense mechanisms against microbial pathogens. Therefore, it has been the aim of many researchers in this area to develop selective complement inhibitors.

Another approach to prevent endothelial damage and inflammation is the use of selectin inhibitors. Selectins are involved in the cooperative multistep process of leukocyte trafficking from blood vessels into sites of inflammation (Rosen, S.D., and Bertozzi, C.R., (1994). The selectins and their ligands. *Curr. Opin. Cell Biol.* **6**, 663-673). The selectins share the

ability to recognize the tetrasaccharide $SiaLe^x$ (sialyl Lewis X). The binding affinities of monomeric $SiaLe^x$ and its mimetics are poor, being in the millimolar range. However, multimeric negatively charged molecules such as heparin, inositol hexaphosphate, sulfatide, and especially the polysaccharide fucoidan are known to be P-selectin inhibitors (Skinner et al. (1989) Characterization of human platelet GMP-140 as a heparin-binding protein. *Biochem. Biophys. Res. Communs* **164**, 1373-1379; Marinier et al. Sulfated galactocerebrosides as potential antiinflammatory agents. *J. Med. Chem.*, 1997, **40**, 3234-3247; Cecconi et al. Inositol polyanions. Noncarbohydrate inhibitors of L- and P-selectin that block inflammation. *J. Biol. Chem.*, 269, 15060-15066 (1994)).

Ushakova et al. (Vopr. Med. Khim, 45:5, 375-383, 2000) describes an inhibitory activity of monomeric and polymeric selectin ligands. According to these authors ($SiaLe^x$, $SiaLe^a$, and HSO_3Le^x) tetrasaccharides, their conjugates with polyacrylamide and a number of further substances block selectins similar to the polysaccharide fucoidan. PAA conjugates containing the ligand tyrosine-O-sulfate in addition to the above mentioned oligosaccharides were the most potent of the tested synthetic selectin blockers. In comparison to the most potent known inhibitor fucoidan, the biligand glycoconjugate HSO_3Le^a-PAA-sTyr displayed similar activity in blocking L-selectin, while its activity towards P-selectin was 10 times lower. All tested synthetic polymers were able to inhibit neutrophil extravasation to inflammation sites in a concentration of about 10 mg/kg.

The manufacturing of glycosylated, in particular sialylated, PAA conjugates is very expensive. The cost of producing a substance such as $SiaLe^x$-PAA lies around 10.000 EUR/100 mg.

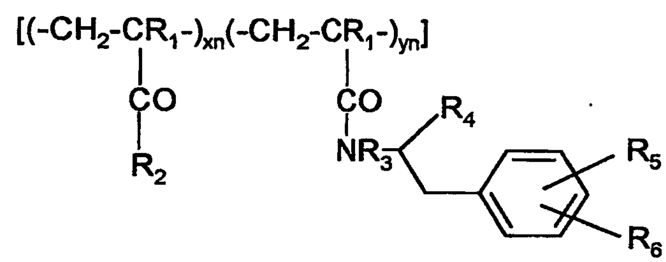
Therefore, there is a need for cost effective substances that selectively inhibit cellular, in particular complement-mediated endothelial cell damage mediated by the patient's natural immunity, such as the complement system, without having systemic effects on blood coagulation.

In particular, there is a need for treating and/or preventing inflammatory reactions towards endothelial cells, especially in a number of diseases such as arteriosclerosis, chronic heart failure, cardiac or brain infarction, surgery related ischemia, vascular rejection reactions, and sepsis, acute respiratory distress syndrome (ARDS), septic shock. Similarly, reactions

mediated by the innate immune system (comprising the complement, coagulation, and kinin systems, natural antibodies, phagocytic, dendritic and NK cells) are involved in the pathogenesis of allogeneic or xenogeneic islet cells rejection as well as in HIV infection.

Disclosure of the invention

One aspect of the present invention provides new polyacrylamide conjugates of the general formula I,



(I)

wherein

R_1 denotes hydrogen or methyl,

R_2 denotes $N(R_7R_8)$ or OH,

R_3 denotes a hydrogen, $C_{1\text{-}6}$ alkyl or $C_{3\text{-}6}$ cycloalkyl,

R_4 denotes H or COO^-M^+,

R_5, R_6 denote, in each case independently of one another a hydrogen, $SO_3^-M^+$ or $OSO_3^-M^+$,

R_7, R_8 denote, in each case independently of one another, hydrogen, $C_{1\text{-}6}$ alcohol, $C_{1\text{-}6}$ alkyl, phenyl, benzyl, phenethyl or $N(R_7R_8)$ denotes a $N(CH_2)_{2\text{-}6}$ ring that may also be substituted,

n is 20 to 500,

y is from 0.2 to 1.0,

x is $1 - y$,

M^+ is a physiologically acceptable monovalent cation.

and their diastereomers or enantiomers in the form of their acids or salts of physiologically compatible bases.

Preferably, in said polyacrylamide conjugate R_1 denotes hydrogen.

In a preferred embodiment said polyacrylamide conjugate comprises a substituent R_2 which denotes $N(R_7R_8)$.

Furthermore, it is also preferred that the substituent R_3 in the above formula denotes hydrogen.

In a preferred embodiment, the present invention relates to polyacrylamide conjugates, wherein R_4 denotes COO^-M^+.

In a further preferred embodiment, the present invention relates to polyacrylamide conjugates, wherein R_6 is hydrogen and R_5 is $SO_3^-M^+$ or $OSO_3^-M^+$ in the meta or para position, preferably in the para position, most preferably R_5 is $OSO_3^-M^+$ in the para position.

In a more preferred embodiment R_5 and R_6 both denote hydrogen.

Preferably, R_7 is hydrogen and R_8 is a C_{1-6} alcohol, more preferably a C_{1-4} alcohol, most preferably ethyl alcohol.

Polyacrylamide conjugates according to the invention comprise a counterion M^+, which may be any suitable pharmaceutically acceptable cation, preferably selected from the group of Na^+, K^+, NH_4^+, Et_3NH^+, $HO(CH_2)_2NH_3^+$.

Polyacrylamide conjugates according to the invention preferably comprise 20 to 400 of the units (n) shown in the above formula. More preferably n is 20 to 300, or even 20 to 100, and most preferably about 20 to 80.

The ratio of the components x and y in the above formula is preferably such that y is 0.2 to 0.8, more preferably 0.3 to 0.6 or even 0.3 to 0.5, most preferably 0.35 to 0.45.

The $SiaLe^x/SiaLe^a$-free polyacrylamide conjugates bearing highly dense sulfated tyrosine or tyramine according to the present invention are excellent site specific blockers of complement activation and P-selectin mediated leucocyte adhesion *in vitro* and *in vivo*, being even more active *in vitro* than fucoidan or low molecular weight dextran sulfate (DXS), the most potent known inhibitors up to now. They interfere much less with blood coagulation than the latter substances. They protect endothelial cells from complement-mediated cytotoxicity. Although the present invention is by no means bound by theory, it seems that that endothelial cell protection of these substances is due to the at least functional replacement of the natural heparan sulfate proteoglycans that are shed from the cell surface upon damage-induced activation of the endothelial cells.

The polyacrylamide conjugates according to the present invention are excellent compounds of choice for inhibiting complement activation and P-selectin mediated leukocyte adhesion *in vitro*. They are preferably employed in basic scientific research as well as in the development of new medicaments and therapies for diseases in which the innate immune system, including complement, plays a major pathophysiological role.

Furthermore, the polyacrylamide conjugates according to the present invention are excellent compounds of choice for protecting endothelial cells from complement-mediated cytotoxicity. In a preferred embodiment the present invention relates to a method for protecting endothelial cells from complement-mediated cytotoxicity comprising the addition of at least one polyacrylamide conjugate according to the present invention to said cells *in vitro*.

The compounds of the present invention have also demonstrated their efficacy *in vivo*.

Therefore, polyacrylamide conjugates according to the invention are useful as a medicament. Preferably, said compounds are used for the preparation of a medicament for protecting endothelial cells from complement-mediated damage.

Chronic heart failure and arteriosclerosis are diseases that are closely linked to complement-mediated endothelial damage and dysfunction (Gullestad et al. Circulation, January 16, 2001, 220-224, Aukrust et al. Circulation, September 25, 2001, 1494-1500, Buono et al. Circulation, June 25, 2002, 3025-3031) The compounds of the present invention are preferably used for the preparation of a medicament for the prevention and/or treatment of inflammatory reactions towards endothelial cells, preferably in arteriosclerosis or chronic heart failure.

In effecting treatment of a subject suffering from diseases associated with complement-mediated damage the compounds disclosed by the present invention for said purpose can be administered in any form or mode which makes the therapeutic compound bioavailable in an effective amount, including oral or parenteral routes. For example, products of the present invention can be administered intraperitoneally, intranasally, buccally, topically, orally, subcutaneously, intramuscularly, intravenously, transdermally, rectally, and the like. One skilled in the art of preparing formulations can readily select the proper form and mode of administration depending upon the particular characteristics of the product selected, the disease or condition to be treated, the stage of the disease or condition, and other relevant circumstances. (Remington's Pharmaceutical Sciences, Mack Publishing Co. (1990)). The products of the present invention can be administered alone or in the form of a pharmaceutical preparation in combination with pharmaceutically acceptable carriers or excipients, the proportion and nature of which are determined by the solubility and chemical properties of the product selected, the chosen route of administration, and standard pharmaceutical practice. For oral application suitable preparations are in the form of tablets, pills, capsules, powders, lozenges, sachets, cachets, suspensions, emulsions, solutions, drops, juices, syrups, while for parenteral, topical and inhalative application suitable forms are solutions, suspensions, easily reconstitutable dry preparations as well as sprays. Compounds according to the invention in a sustained-release substance, in dissolved form or in a plaster, optionally with the addition of agents promoting penetration of the skin, are suitable percutaneous application preparations. The products of the present invention, while effective themselves, may be formulated and administered in the form of their pharmaceutically ac-

ceptable salts, such as acid addition salts or base addition salts, for purposes of stability, modulation of hydrophobicity, increased solubility, and the like.

The amount of active agent to be administered to the patient depends on the patient's weight, on the type of application, symptoms and the severity of the illness. Normally, 0.1 mg/kg to 25 mg/kg of at least one substance of the general formula I is administered, but when applied locally, e.g. intracoronary administration, much lower total doses are also possible.

In a preferred embodiment, the compounds of the present invention are useful for the preparation of a medicament for preventing ischemia/reperfusion damage.

In another preferred embodiment, the present invention relates to the use of said compounds for the preparation of a medicament for the treatment of cardiac or brain infarction.

Preferably, polyacrylamide conjugates according to the invention are used for the preparation of a medicament for preventing damage to organs during surgery-related ischemia, more preferably for the preparation of a medicament for preventing acute vascular rejection reactions.

More preferably, polyacrylamide conjugates according to the invention are used for the preparation of a medicament for preventing acute vascular rejection reactions in ABO-incompatible transplants or xenotransplants.

Due to its protective properties with respect to complement-mediated damage, the compounds of the present invention are particularly useful for the preparation of solutions and/or gels for safe-keeping of life donor organs for use in transplants.

More preferably, the compounds of the present invention are useful for the preparation of a medicament for use in allogeneic and xenogeneic islet transplantation.

Recently, sulfated polymers have entered clinical trials for the prevention and treatment of HIV such as Carregard, a seaweed extract, PRO 2000, a naphtalene sulfate polymer and dextrin-2-sulfate, all being polymers containing negatively charged molecules that block the

electrostatic attraction between HIV's gp 120 envelope protein and target cells (Julia Clayton, BioMedNet News, May 16 2002). In this context, although not bound by theory, the compounds of the present invention are deemed particularly useful for the preparation of a medicament for use in the prevention and/or treatment of HIV infection.

In another embodiment the present invention relates to the use of a polyacrylamide conjugate of the invention for the preparation of a medicament for use in the prevention and/or treatment of severe sepsis, acute respiratory distress syndrome (ARDS), or septic shock.

Abbreviations: SiaLe^x = sialyl Lewis X, Neu5Acα2-3Galß1-4(Fucα1-3)GlcNAc; SiaLe^a = sialyl Lewis A, Neu5Acα2-3Galß1-3(Fucα1-4)GlcNAc; HSO_3Le^a = 3'-sulfo-Lewis A; 3'-HSO_3Galß1-3(Fucα1-4)GlcNAc; sTyr = tyrosine-O-sulfate; PAA = polyacrylamide

Figures

Fig. 1 demonstrates the inhibitory potency of five sTyr-PAA conjugates which differ in their sTyr loading in comparison to the polysaccharide fucoidan. The *in vitro* test-system is based on the inhibition of HSO_3Le^a-PAA-biotin binding to recombinant P-selectin. For details, see example 1.

Fig. 2 shows the effects of different PAA-conjugates as well as DXS (dextran sulfate) on complement activation in a standard hemolytic assay (CH50 test) with antibody-sensitized sheep erythrocytes.
Dose-response curves for the selected substances are provided in comparison to DXS. The sTyr-PAA conjugate with a 40% substitution rate was the best complement inhibitor of all substances tested, demonstrating a stronger inhibitory activity than DXS.

Fig. 3 shows the effect of DXS (dextran sulfate) as well as PAA-sTyr on complement and coagulation.
The figure demonstrates that DXS already leads to a prolongation of the aPTT (filled circles) to more than 300 seconds at a concentration of 0.05 mg/ml, with only a minimal inhibition of complement activation (empty circles) at that concentration. In contrast, PAA-sTyr (40%) shows only a slight prolongation of the aPTT (filled squares) at 0.05 mg/ml, and reduces complement activation to 27% at that concentration (empty squares). Therefore,

PAA-STyr with a 40% substitution rate was both, a better complement inhibitor than DXS and a much less potent anti-coagulant than the latter.

Examples

The following examples further illustrate the best mode contemplated by the inventors for carrying out their invention.

Example 1 Inhibition of P-selectin mediated leukocyte adhesion and extravasation

Reagents

All PAA-based neoglycoconjugates were from 30 to 40 kDa and obtained according to standard methods (Gordeeva, E.A., Tuzikov, A.B., Galanina, O.E., Pochechueva, T.V. & Bovin, N.V. (2000). Microscale synthesis of glycoconjugate series and libraries. *Anal. Biochem.* **278**, 230-232; Bovin, N.V., Korchagina, E.Yu., Zemlyanukhina, T.V., Byramova, N.E., Galanina, O.E., Zemlyakov, A.E., Ivanov, A.E., Zubov, V.P., and Mochalova, L.V. (1993) Synthesis of polymeric neoglycoconjugates based on N-substituted polyacrylamide. *Glycoconj. J.* **10**, 142-151). sTyr-PAA (containing 80 mol % sTyr) was synthesized using 200% excess of sTyr in relation to the activated polymer. The degree of sTyr substitution was determined by the conjugate's increase in weight. Recombinant ZZ-selectin (monovalent) lacking the transmembrane and cytosolic domains was produced as C-terminal chimera with the ZZ domain of protein A (Priest, R., Nawaz, S., Green, P.M., and Bird, M.I. (1995) Adhesion of eosinophils to E- & P-selectin. *Biochem. Soc. Trans.* **23**, 162S). Tripeptide Tyr-Tyr-Tyr (Bachem, Germany) and aminoglucitol (Sigma) were per-O-sulfated with SO_3/Py complex (Field, R.A.; Otter, A.; Fu, W.; Hindsgaul, O. Carbohydr. Res. 1995, 276, 347-363). Fucoidan was purchased from Sigma (USA).

In vitro assay for inhibition of P-selectin binding

The assay was performed on 96-Well plates. Human IgG was used as a primary coating reagent to immobilize recombinant selectin via the ZZ-domain of the fusion protein. The working concentration of selectin was 3 ng/well. HSO_3Le^a-PAA-biotin (Zemlyanukhina, T.V., Nifant'ev, N.E., Shashkov, A.S., Tsvetkov, Y.E., and Bovin, N.V. (1995) Selectin receptors:

synthesis of spacer-armed sulfated trisaccharides Lewis A and Lewis X and neoglycoconjugates thereof. *Carbohydrate Lett.* **1**, 277-284) was selected from HSO_3Le^a-, HSO_3Le^x-, $SiaLe^a$-, and $SiaLe^x$-PAA because it showed a maximum level of binding in this assay. Details of this assay were published in (Game, S.M., Rajapurohit, P.K., Clifford, M., Bird, M.I., Priest, R., Bovin, N.V., Nifant'ev, N.E., O'Beirne, G., and Cook, N.D. (1998) Scintillation proximity assay for E-, P-, and L-selectin utilizing polyacrylamide-based neoglycoconjugates as ligands. *Anal. Biochem.* **258**, 127-135; Tatyana V. Pochechueva, Oxana E. Galanina, Michael I. Bird, Nikolay E. Nifant'ev, Nicolai V. Bovin. Assembly of P-Selectin Ligands on a Polymeric Template, Chem&Biol, Vol. 9, 2002, p. 757-762; Natalia Ushakova, Marina Preobrazhenskaya, Mike I. Bird, Richard Priest, A.V. Semenov, A.V. Mazurov, Nikolay Nifant'ev, Tatyana Pochechueva, Oxana Galanina, Nicolai Bovin. Monomeric and Multimeric Blockers of Selectins: Comparison of *In Vitro* and *In Vivo* Activity/ Biol. Chem. Hoppe Seyler, 2002 submitted).

Rat peritoneal inflammation model

Acute rat peritonitis was induced as described in (Preobrazhenskaya, M.E., Berman, A.E., Mikhailov, V.I., Ushakova, N.A., Mazurov, A.V., Semenov, A.V., Usov, A.I., Nifant'ev, N.E., and Bovin, N.V. (1997) Fucoidan inhibits leukocyte recruitment in a model peritoneal inflammation in rat and blocks interaction of P-selectin with its carbohydrate ligand. *Biochem. Mol. Biol. Int.* **43**, 443-451) with some modifications. 10 ml of PBS containing 10% peptone was injected intraperitoneally into female Wistar rats (180-220 g). After 3 h rats were sacrificed and their peritoneal cavities were laved with 30 ml PBS containing 60 units/ml of heparin, 0.02% EDTA and 0.03% bovine serum. The total cell number in the lavage was counted and the cell suspension was concentrated by centrifugation at 400g for 10 min. After 1:1 dilution with bovine serum smears were prepared and stained according to the Pappenheim method. Neutrophils were counted on two parallel slides and the total neutrophil number was calculated. The inhibitors were administered intravenously as a single dose in 0.3 ml sterile 0.9% NaCl 15 min after peptone injection. The same volume of NaCl solution was injected into control animals. A difference between the control and tested groups was analyzed for statistical significance based on Student's *t*-test, *p*-values <0.05 were considered to be significant.

Results of the in vitro P-selectin inhibition assay

According to previous data on synthetic multivalent inhibitors, (e.g. Tatyana V. Pochechueva, Oxana E. Galanina, Michael I. Bird, Nikolay E. Nifant'ev, Nicolai V. Bovin. Assembly of P-Selectin Ligands on a Polymeric Template. Chem&Biol, Vol. 9, 2002, p. 757-762) as well as those documents cited below, synthetic manipulations of a molecules charge will influence its selectin-binding properties as much as the optimization of a carbohydrate ligand. The polyacrylamide conjugate $SiaLe^a$ inhibits P-selectin with an IC_{50} of 40 μM (by $SiaLe^a$ residue) or 0.4 μM for the whole macromolecule (Tatyana V.Pochechueva, Oxana E.Galanina, Michael I. Bird, Nikolay E. Nifant'ev, Nicolai V.Bovin. Assembly of P-Selectin Ligands on a Polymeric Template. Chem&Biol, Vol. 9, 2002, p. 757-762). In comparison, sulfatide (3-O-sulfated galactocerebroside) and some of its synthetic analogues demonstrate an IC_{50} of 0.1-1 μM (A. Marinier, A. Martel, J. Banville, C. Bachand, R. Remillard, R. Remillard, P. Lapointe, B. Turmel, M. Menard, W.E. Harte Jr., J.J.K. Wright, G. Todderud, K.M. Tramposch, J. Bajorath, D. Hollenbaugh, A. Aruffo. Sulfated galactocerebrosides as potential antiinflammatory agents. *J. Med. Chem.*, 1997, **40**, 3234-3247). For the highly sulfated polysaccharide fucoidan from algae, which is known to be the most potent among those described, the IC_{50} is 0.1 μM (it is a regular polysaccharide with repeating hexasaccharide units) (Tatyana V. Pochechueva, Oxana E. Galanina, Michael I. Bird, Nikolay E. Nifant'ev, Nicolai V. Bovin. Assembly of P-Selectin Ligands on a Polymeric Template Chem&Biol, 2002, submitted; Ley, K., Linnemann, G., Meinen, M., Stoolman, L.M., and Gaethgens, P. (1993) Fucoidin, but not yeast polyphosphomannan PPME, inhibits leukocyte rolling in venules of the rat mesentery. *Blood* **81**, 177-185).

High-affinity binding of P-selectin with PSGL-1 requires the latter to contain both, a carbohydrate chain with $SiaLe^x$ and sulfated tyrosine residues on the polypeptide chain. It was surprisingly demonstrated that an sTyr-based monoligand conjugate possesses a P-selectin binding activity that is comparable to that of PSGL-1 and fucoidan. Because the three sulfotyrosine residues in PSGL-1 appear to be adjacent, i.e. $sTyr_{46}$-X-$sTyr_{48}$-X-X-$sTyr_{51}$ (A. Leppanen, S.P.White, J. Helin, R.P. McEver, R.D. Cummings. Binding of glycosulfopeptides to P-selectin requires stereospecific contributions of individual tyrosine sulfate and sugar residues J. Biol. Chem, 275, 39569-39578 (2000)), those mimetics of the present invention were designed to provide a maximum or near maximum density of sulfated residues. Two low molecular weight compounds, sTyr-sTyr-sTyr (#13, Table 1) and hexa-O-sulfo-aminoglucitol (#14) were also synthesized, where the density of HSO_3 groups was

rather high, in particular in the second one. However, both compounds were unable to inhibit the recombinant P-selectin in the cell-free assay (Table 1, see below).

On the other side, multivalent compounds such as the PAA conjugates with a high sTyr loading display a dramatic increase in P-selectin-blocking potency (Fig. 1). A twofold sTyr loading increase in the conjugates containing sTyr (in range 5 to 80 mol %) gave a tenfold higher activity. The most potent inhibitor was the 80 mol-% sTyr conjugate (higher loading is problematic but possible) which had an IC_{50} value of 10 nM for one sTyr residue (i.e. ~ 0.1 nM if counted for the whole macromolecule) or 6 ng/ml in weight. This highly sulfated conjugate was a significantly more potent inhibitor of P-selectin than PSGL-1 and fucoidan.

Of importance for a higher activity is not only a high negative charge of the molecule, but also the origin of an anion group as well as its proximity. Three results obtained *in vitro* confirm this finding. First, the conjugate HSO_3Le^a-PAA-sTyr (15/5 = the polyacrylamide containing 155 mol HSO_3Le^a and 5 % mol sTyr) (see substance 6 in table 1 below) with a total amount of sulfogroups being the same as for sTyr-PAA (20) appeared to be sixty times less active than the latter. Secondly, the PAA-derivative of $HSO_3OCH_2CH_2$- (see substance 9 in table 1 below) is less active by three orders of magnitude when compared to the corresponding derivative of sTyr (see substance 1 in table 1 below), i.e. the sTyr-PAA – P-selectin interaction is not exclusively Coulomb in nature. Instead, the aromatic tyrosine residue is important, too. Finally, Table 1 presents data for a different polyanion, being just the $SiaLe^a$-conjugate of a polyacrylic acid. According to Table 1 and Fig. 1 the derivative of a polyacrylic acid $SiaLe^a$-pA (see substance 3 in table 1 below), containing 20 mol% oligosaccharide and 80 mol% of carboxyl groups is ten times less potent than a similar compound based on sulfotyrosine, i.e. $SiaLe^a$-PAA-sTyr (see substance 5 in table 1 below).

Thus, it was surprisingly found that for reaching high-affinity binding to P-selectin, a polymeric molecule may lack a carbohydrate ligand, but must contain a high content of sulfotyrosine. The interaction stoichiometry of sTyr-PAA binding with P-selectin in the solid phase assay seems to be 1:1, indicating that the high activity is not the result of cross-binding between one molecule of sTyr-PAA with several P-selectin molecules. Although the present invention is not bound by theory, it seems that due to the high density of sTyr residues in the conjugates of the present invention, these compounds bind to all of the three positively charged amino acid residues in the sTyr-binding site and due to the sufficient size of the

conjugate the lectin site of P-selectin is shielded sterically. Due to the spatial proximity, it cannot be exclude that some of the sTyr residues interact with the lectin site, i.e. with the arginine which should bind to SiaLe^x carboxyl. The above mentioned model explains both, the need for high density of sTyr and the need for a large molecular size of the compound that promotes the blocking of simultaneous binding to the two distinct sites of P-selectin.

Table 1 shows the inhibition of HSO_3Le^a-PAA-biotin binding with P-selectin by monomeric and polymeric compounds

INHIBITOR	IC_{50}, µM[1]
Multimeric	
1. sTyr-PAA (80)	0.01 (6 ng/ml)
2. Fucoidan	0.1 (100 ng/ml)[2]
3. SiaLe^a-polyacrylic acid	2
4. SiaLe^a-PAA-sTyr (20/10)[3]	10
5. SiaLe^a-PAA-sTyr (20/max)[4]	0.1
6. HSO_3Le^a-PAA-sTyr (15/5)	25
7. SiaLe^a-PAA	40
8. HSO_3Le^a-PAA	120
9. $HSO_3OCH_2CH_2$-PAA (80)	40 (6 µg/ml)
Monomeric	
10. SiaLe^a, SiaLe^x	>1500
11. HSO_3Le^a	NI (1.5 mM)
12. sTyr	NI
13. sTyr-sTyr-sTyr	NI
14. Hexa-O-sulfo-aminoglucitol	NI

[1] The Values for the inhibition were the means of at least triplicate determinations. Standard deviations were less than 10%.

[2] The molar concentration calculation is based on an assumption that the hexasaccharide is an active unit.

[3]The figure in brackets designates the mol-% of the given ligand in the polyacrylamide conjugate; two figures separated via "/" means two ligands; if no sign it is 20 mol-%.

[4]"max" means that after introduction of 20 mol-% carbohydrate ligand to the polymer it was subjected to action of excess sTyr resulting in maximum possible content of sTyr, which in reality was about 60%.

Results of rat peritonitis model

Peptone-induced acute rat peritonitis was characterized by neutrophil extravasation into the peritoneal cavities. Intravenous injection of the inhibitors resulted in different degrees of inhibition of neutrophil extravasation. Table 2 (see below) provides the results of this assay. According to the results only fucoidan and three of the tested synthetic substances according to the invention definitely blocked the inflammation process. sTyr-PAA (80 mol%) demonstrated a dramatic activity *in vitro,* while it turned out to be a little less active than fucoidan (Table 2) in the rat *in vivo* model. This result reflects that P-selectin binding can be completely blocked *in vitro* while *in vivo* there are a number of other mechanisms that promote leukocyte extravasation. Inositol hexaphosphate (O.Cecconi, R.N. Nelson, W.G. Roberts, K. Hanasaki, G. Mannori, C. Schultz, T.R. Ulich, A. Aruffo, M.P. Bevilacqua. Inositol polyanions. Noncarbohydrate inhibitors of L- and P-selectin that block inflammation. *J. Biol. Chem.*, 269, 15060-15066 (1994)) was yet less active in a mouse test system inhibiting the yield of neutrophils (almost half of them) in the dose about 70 mg/kg. A natural ligand such as recombinant PSGL-1 acts in a dose of about 1 mg/kg (S.P. Khor, K. McCarthy, M. Dupont, K. Murray, G. Timony. Pharmacokinetics, pharmacodynamics, allometry, and dose selection of rPSGL-Ig for phase I trial. *J. Pharmacol. Exp. Ther.*, 293, 618-624 (2000);R. Hayward, B. Campbell, Y.K. Shin, R. Scalia, A.M. Lefer. Recombinant soluble P-selectin glycoprotein ligand-1 protects against myocardial ischemic reperfusion injury in cats. *Cardiovascular Res.*, 41, 65-76 (1999)) which approaches the fucoidan activity under study. The low activity of synthetic high molecular weight blockers of P-selectin was observed before (Natalia Ushakova, Marina Preobrazhenskaya, Mike I. Bird, Richard Priest, A.V. Semenov, A.V. Mazurov, Nikolay Nifant'ev, Tatyana Pochechueva, Oxana Galanina, Nicolai Bovin. Monomeric and Multimeric Blockers of Selectins: Comparison of *In Vitro* and *In Vivo* Activity/ Biol. Chem. Hoppe Seyler, 2002 submitted). To reach an appropriate *in vivo* inhibition, even strong substance-selectin binding does not seem sufficient. Additional properties seem to be of importance, too, i.e. the ability of rolling as well as tropism to the corresponding target cells and at the same time a minimum binding to other cells.

Table 2 shows the degree of inhibition of rat peritonitis by sialylated and sulfated PAA-conjugates in comparison with fucoidan and free SiaLe^x tetrasaccharide. The data are presented as means ± SEM.

Preparation	Number of rats in group	Number of neutrophils per rat x 10^{-6}	Mean inhibition (% to control)	Dose, mg per rat
Series 1				
Control group (no preparation)	12	41.4±5.4		-
SiaLe^a-PAA-sTyr (20/10)	8	19.0±3.8	54 ($p<0.01$)	1.5-3.0
SiaLe^x	11	37.0±5.6	11	1.0-3.0
SiaLe^x-PAA	9	30.0±6.2	27	1.0
Series 2				
Control group (no preparation)	19	32.7±2.9		-
sTyr-PAA (80)	5	11.4±0.6	65 ($p<0.001$)	2.0
sTyr-PAA (80)	5	23.5±3.8	28	1.0
Fucoidan	10	2.5±0.5	92 ($p<0.001$)	1.0
$HSO_3OCH_2CH_2$-PAA (80)	5	17.4±5.8	47 ($p<0.05$)	2.0

Example 2: Effect of different PAA-conjugates as well as DXS (dextran sulfate) on complement activation and cytotoxicity

Effect of different PAA-conjugates on activation of the classical complement pathway (CH50 test)

PAA-conjugates of several different phosphorylated or sulfated sugars and amino acids were tested for inhibition of complement activation in a standard hemolytic assay (CH50 test) with antibody-sensitized sheep erythrocytes. The tested substances were alpha-D-Man-6-phosphate-PAA (20%), beta-D-Gal-3-sulfate-PAA (20%), and sTyr-PAA (40%). Fresh human serum was mixed with serial dilutions of the inhibitors (DXS as well as the

different PAA-conjugates) at a final serum concentration of 0.5% and immediately incubated with erythrocytes for 1 h at 37°C in a shaking water bath. After centrifugation hemolysis was determined by measuring the absorption of the supernatant at 414 nm.

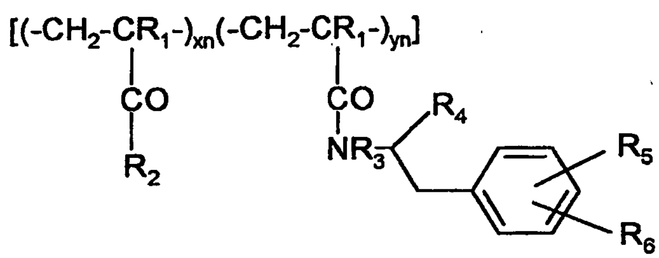
Dose-response curves for the selected substances, in comparison to DXS (dextran sulfate), are given below in Fig. 2. The sTyr-PAA conjugate with a 40% substitution rate was the best complement inhibitor of all substances tested, demonstrating a stronger inhibitory activity than DXS.

Effect of DXS (dextran sulfate) as well as PAA-sTyr on complement and coagulation

Several different variants of PAA-conjugates were tested first in a CH50 assay for complement inhibition, and good complement inhibitors were then further tested in an aPTT assay (activated partial thromboplastin time, a standard assay for coagulation) for their effect on the coagulation system.
The CH50 test for evaluation of complement inhibition (see left panel of Fig. 3) was performed as described for figure 2. To check the effect of the substances on coagulation they were added as serial dilutions to human plasma (final concentration 50%) and the coagulation time of the mixtures was measured in a standard aPTT assay (see right panel of Fig. 3). Fig. 3 shows that DXS already leads to a prolongation of the aPTT (filled circles) to more than 300 seconds at a concentration of 0.05 mg/ml, with only a minimal inhibition of complement activation (empty circles) at that concentration. In contrast, PAA-STyr (40%) shows only a slight prolongation of the aPTT (filled squares) at 0.05 mg/ml, and reduces complement activation to 27% at that concentration (empty squares). Therefore, PAA-STyr with a 40% substitution rate was both a better complement inhibitor than DXS and a much less potent anti-coagulant than the latter.

Claims:

1. Polyacrylamide conjugate of the general formula I,



(I)

wherein

R_1 denotes hydrogen or methyl,

R_2 denotes $N(R_7R_8)$ or OH,

R_3 denotes a hydrogen, C_{1-6} alkyl or C_{3-6} cycloalkyl,

R_4 denotes H or COO^-M^+,

R_5, R_6 denote, in each case independently of one another a hydrogen, $SO_3^-M^+$ or $OSO_3^-M^+$,

R_7, R_8 denote, in each case independently of one another, hydrogen, C_{1-6} alcohol, C_{1-6} alkyl, phenyl, benzyl, phenethyl or $N(R_7R_8)$ denotes a $N(CH_2)_{2-6}$ ring that may also be substituted,

n is 20 to 500,

y is from 0.2 to 1.0,

x is 1 - y

M^+ is a physiologically acceptable monovalent cation.

and their diastereomers or enantiomers in the form of their acids or salts of physiologically compatible bases.

2. Polyacrylamide conjugate of claim 1, characterized in that R_1 denotes hydrogen.

3. Polyacrylamide conjugate of claim 1 or 2, characterized in that R_2 denotes $N(R_7R_8)$.

4. Polyacrylamide conjugate according to any of claims 1 to 3, characterized in that R_3 denotes hydrogen.

5. Polyacrylamide conjugate according to any of claims 1 to 4, characterized in that R_4 denotes COO^-M^+.

6. Polyacrylamide conjugate according to any of claims 1 to 5, characterized in that R_6 is hydrogen and R_5 is $SO_3^-M^+$ or $OSO_3^-M^+$ in the meta or para position, preferably in the para position, most preferably R_5 is $OSO_3^-M^+$ in the para position.

7. Polyacrylamide conjugate according to any of claims 1 to 5, characterized in that R_5 and R_6 both denote hydrogen.

8. Polyacrylamide conjugate according to any of claims 1 to 7, characterized in that R_7 is hydrogen and R_8 is a C_{1-6} alkohol, preferably a C_{1-4} alkohol, most preferably ethyl alcohol.

9. Polyacrylamide conjugate according to any of claims 1 to 8, characterized in that the counterion M^+ is selected from the group of Na^+, K^+, NH_4^+, Et_3NH^+, $HO(CH_2)NH_3^+$.

10. Polyacrylamide conjugate according to any of claims 1 to 9, characterized in that n is 20 to 400, preferably 20 to 300, more preferably 20 to 100, most preferably about 20 to 80.

11. Polyacrylamide conjugate according to any of claims 1 to 10, characterized in that y is 0.2 to 0.8, preferably 0.3 to 0.6, more preferably 0.3 to 0.5, most preferably 0.35 to 0.45.

12. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for inhibiting P-selectin *in vitro*.

13. A method for protecting endothelial cells from complement-mediated cytotoxicity comprising the addition of a polyacrylamide conjugate according to any of claims 1 to 11 to said cells *in vitro*.

14. Polyacrylamide conjugate according to any of claims 1 to 11 for use as a medicament.

15. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for protecting endothelial cells from complement-mediated cytotoxicity.

16. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for the prevention and/or treatment of inflammatory reactions towards endothelial cells, preferably endothelial cells involved in arteriosclerosis or chronic heart failure.

17. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for preventing ischemia/reperfusion damage.

18. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for the treatment of cardiac or brain infarction.

19. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for preventing damage to organs during surgery-related ischemia.

20. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for preventing acute vascular rejection reactions.

21. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for preventing acute vascular rejection reactions in ABO-incompatible transplantation or xenotransplantation.

22. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of solutions for safe-keeping of life donor organs for use in transplants.

23. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for use in allogeneic and xenogeneic islet transplantation.

24. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for use in the prevention and/or treatment of HIV infection.

25. Use of a polyacrylamide conjugate according to any of claims 1 to 11 for the preparation of a medicament for use in the prevention and/or treatment of severe sepsis, acute respiratory distress syndrome (ARDS), or septic shock.

Abstract

The present invention relates to new non-glycosylated polyacrylamide conjugates, a method for protecting endothelial cells from complement-mediated cellular damage and the use of said non-glycosylated polyacrylamide conjugates as a medicament.

Fig. 1

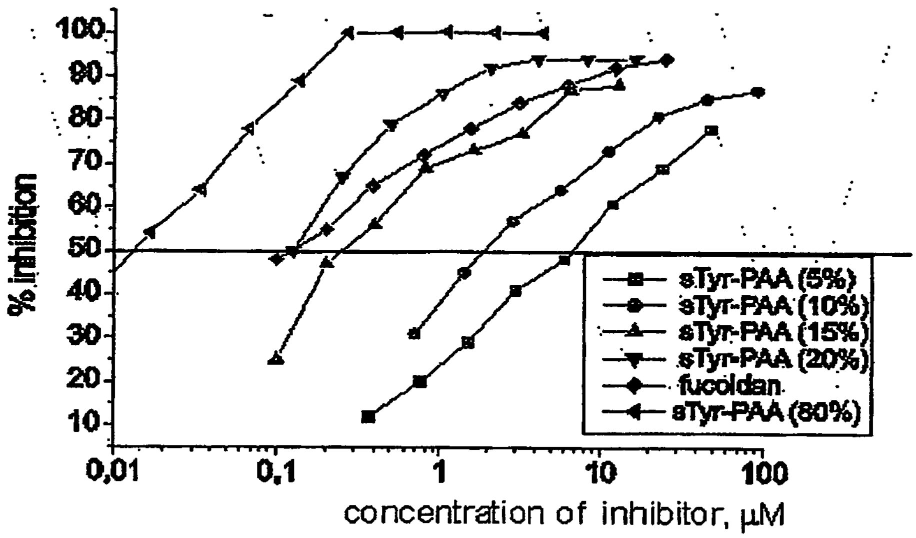

% inhibition
100
90
80
70
60
50
40
30
20
10
0,01
0,1
1
10
100
concentration of inhibitor, µM
sTyr-PAA (5%)
sTyr-PAA (10%)
sTyr-PAA (15%)
sTyr-PAA (20%)
fucoidan
sTyr-PAA (80%)

Fig. 2

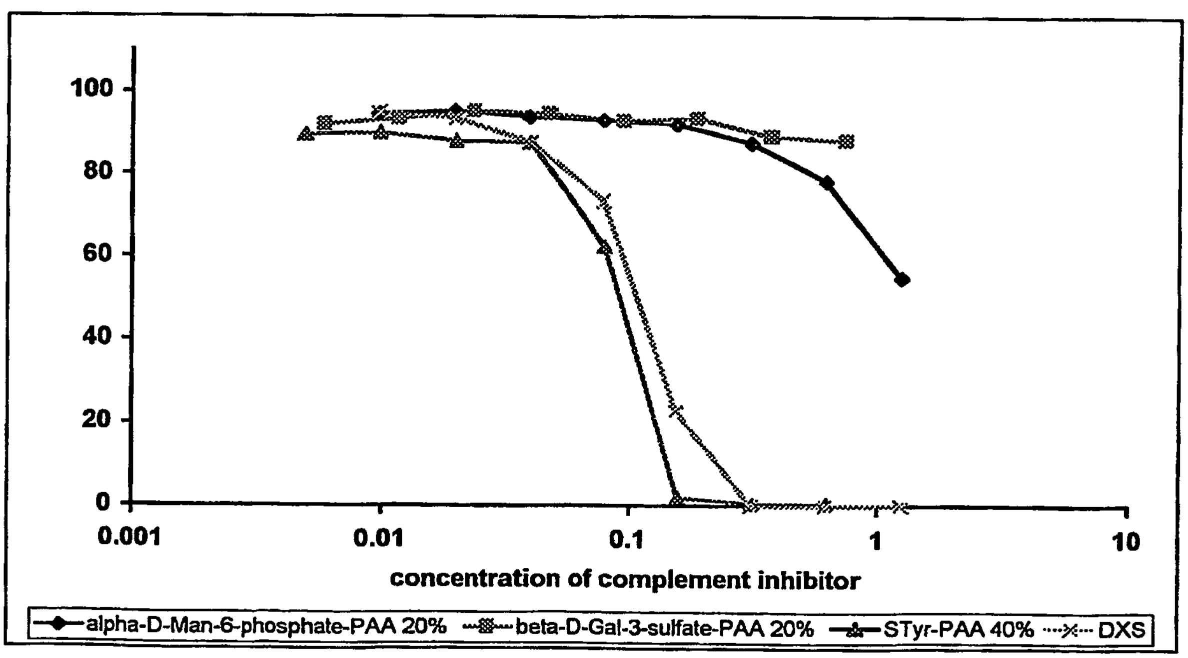
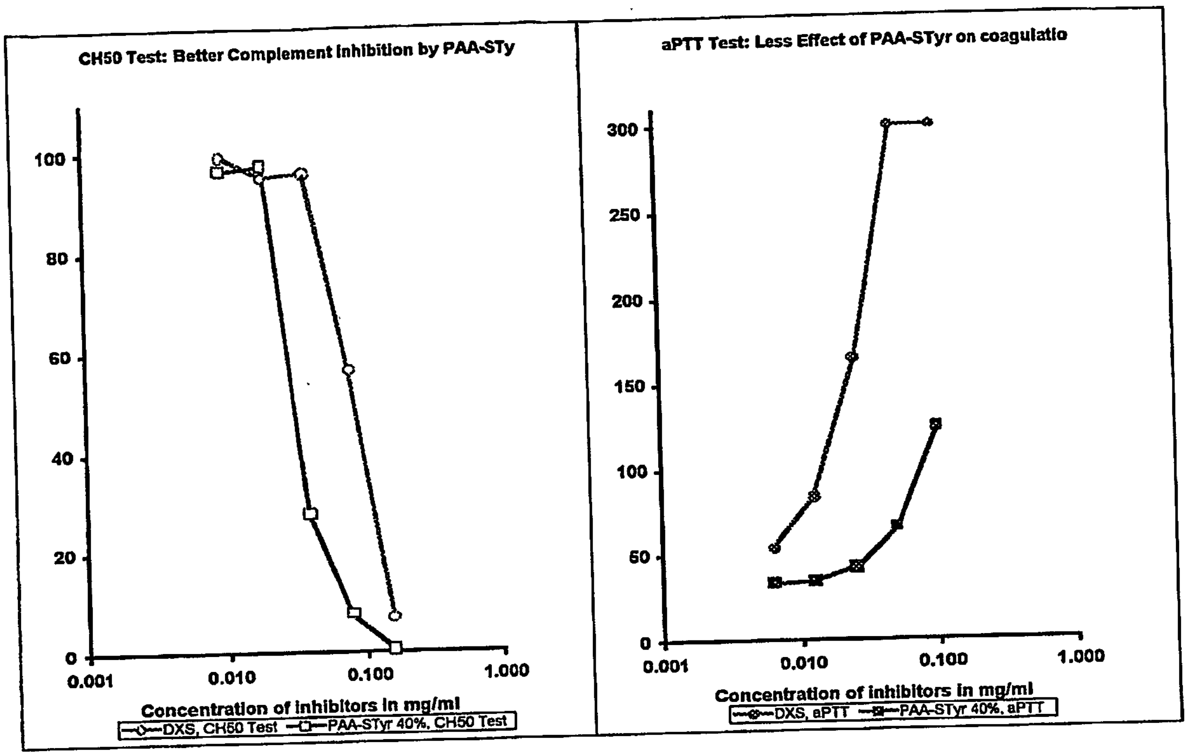

100
80
60
40
20
0
0.001
0.01
0.1
1
10
concentration of complement inhibitor
alpha-D-Man-6-phosphate-PAA 20%
beta-D-Gal-3-sulfate-PAA 20%
STyr-PAA 40%
DXS

Fig. 3


CH50 Test: Better Complement Inhibition by PAA-STy
100
80
60
40
20
0
0.001
0.010
0.100
1.000
Concentration of inhibitors in mg/ml
DXS, CH50 Test
PAA-STyr 40%, CH50 Test
aPTT Test: Less Effect of PAA-STyr on coagulatio
300
250
200
150
100
50
0
0.001
0.010
0.100
1.000
Concentration of inhibitors in mg/ml
DXS, aPTT
PAA-STyr 40%, aPTT